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THE SEROLOGIC RELATIONSHIPS BETWEEN STRAINS OF THE PFEIFFER BACILLUS

INFLUENZA STUDIES. X *

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The importance of determining the essential nature of the various strains of the Pfeiffer bacillus found in epidemic influenza has been clearly set forth by Park and his coworkers.[1] If the strains isolated during the 1918-1920 epidemic were of a single immunologic type and differed from those isolated during interepidemic periods the presumption that a specific strain of this organism was the cause of the pandemic would be greatly strengthened. The majority of recent investigators who have studied this question have failed to find any general antigenic relationship among the strains of epidemic origin.[2] Maitland and Cameron,[3] in a study of 38 strains isolated from hospital patients during a period when no cases of epidemic influenza were seen, found that "nearly all strains of B. influenzae possess a serological individuality as determined by agglutination and agglutinin absorption." On the other hand, Huntoon and Hannum [4] obtained "an almost complete absorption of agglutinin for immunizing and heterologous strains" and found no strains among their collection which did not show "relationship either directly or indirectly through absorption." Small and Dickson [5] also in the study of 10 strains recognized 4 different immunologic groups, and finding that 3 fell in one group and 4 in another, state that "70 per cent. of the strains studied fell into two groups." Chesney,[6] who studied 12 strains of hemoglobinophilic bacilli isolated from influenza patients in 1920, found evidence of a serologic relationship in 4.

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[1] Jour. Am. Med. Assn., 1919, 73, p. 318. Park, W. H., and Williams, A. W.: Am. Jour. Pub. Health, 1919, 34, p. 45. Valentine, E., and Cooper, G.: Jour. Immunol., 1919, 4, p. 569.

[2] Povitzky, Olga, and Denny, Helen: Ibid., 1921, 6, p. 65. Coca, A. F., and Kelley, Margaret: Ibid., 1921, 6, p. 87. Utheim, K.: Jour. Infect. Dis., 1920, 27, p. 460. Bell, H. H.: Ibid., p. 464. Cooke, J. V.: Ibid., p. 476. Anderson, Ruth, and Schultz, O. T.: Jour. Exper. Med., 1921, 33, p. 653. Yabe, S.: Brit. Jour. Exper. Path., 1921, 2, p. 197.

[3] Brit. Jour. Exper. Path., 1921, 2, p. 283.

[4] Jour. Immunol., 1919, 4, p. 167.

[5] Jour. Infect. Dis., 1920, 26, p. 230.

[6] Jour. Infect. Dis., 1921, 29, p. 132.

Our possession of a large number of Pfeiffer bacillus strains isolated at different times and places has given us a favorable opportunity for studying this question. The strains tested have comprised:

13 strains from throats of patients ill with influenza at the Great Lakes Naval Training Station, Jan., 1920.

7 strains from throats of patients ill with influenza at Camp Grant, Rockford, Ill., Jan., 1920.

11 strains from students at the University of Chicago who were suffering from rhinitis, pharyngitis or other respiratory ailment, 1919-1920.

2 strains from University of Chicago patients ill with influenza, 1918.

16 strains from persons apparently in good health and with a normal upper respiratory tract; these strains were isolated between Feb. 15, 1920, and March 31, 1920.

4 strains from patients ill with influenza in New York City, Oct. 1918; obtained through the kindness of Dr. A. W. Williams.

1 strain from sputum of patient ill with influenza, Chicago, Ill., Oct., 1918.

1 strain from lung of patient dying of influenza, Chicago, Ill., Oct., 1918.

1 strain from throat of normal person, New York City, Oct., 1918.

1 strain from cerebrospinal fluid of patient with meningitis; obtained through kindness of Dr. D. J. Davis, Chicago.

Some of these strains were obtained from one and the same individual, but each strain isolated has been kept carefully separate throughout with due precautions as to purity. The agglutinative behavior of the independent strains from a single source will be referred to later.

The method of obtaining an agglutinating serum was essentially similar to that followed by other observers. Rabbits were injected intravenously with a physiologic salt suspension of the Pfeiffer bacillus washed from 24-hour heated blood agar slants. A series of 3 daily injections was followed by a 4 day rest and then the injections were resumed, this procedure usually being repeated for a period of several weeks before a serum with sufficiently high titer was obtained. Heat-killed suspensions were used for the first of these injections. Dosage of living cultures was increased up to one full agar slant. Bleedings were made after rest periods, the blood being aspirated from the heart in amounts of 10-20 c c. The serum was separated after the blood had stood over night.

The serum test mixtures were made by adding to 0.1 c c of the appropriate serum dilution, 0.9 c c of a light suspension of Pfeiffer bacilli, 0.8% salt solution being used for all suspensions and dilutions. These mixtures were placed in a 56 C. water bath and examined after 24 hours and again after 2 days.

It was often, but not always, true that agglutination was as far advanced after 1 day as after 2. The agglutination of the homologous strains is more rapid than that of the heterologous. While the action of agglutinative serums on the homologous strains was almost invariably complete after the first day, heterologous strains were often acted on more slowly. In one instance only about one-fourth of about 50 heterologous strains showed complete agglutination at 1 : 50 or higher (titer 1 : 1,600) after 24 hours, while five-sixths of this number showed agglutination after 2 days.

For one typical experimental series 5 serums were prepared: one from a strain isolated from human lung at necropsy (indol +), one from the meningeal fluid in a case of meningitis (indol +), one from the nasopharynx of an influenza patient (1920) (indol —), one from the nasopharynx of a presumably normal person (1920) (indol +), and one from the nasopharynx of a person with rhinitis (indol —). In general the titer of the serums tested was about 1 : 1,600 (complete agglutination), but in a few instances serums with a titer of 1 : 800-1 : 1,200 were used. With such strengths the majority of the heterologous strains were agglutinated completely in a 1 : 200 dilution, sometimes higher, and occasionally, though rarely, as high as the homologous strains. A careful study of all our tables, which it seems hardly necessary to reproduce here in detail, shows no apparent relation between the origin of the strain and the action of its agglutinogenic serum on other organisms. The strains from influenza cases were not agglutinated in larger numbers or to a greater degree by the serum from an influenza strain than were those strains obtained from normal individuals. Strains from pathologic lesions yielded serum which agglutinated strains from the nasopharynx of healthy persons no more and no less than did serum from animals inoculated with "normal" strains. Indol-producing strains gave serum which agglutinated indol-producing and nonindol-producing strains without discrimination. The reaction that was most constant was that the culture used to produce the serum would be agglutinated more rapidly and in higher dilution than any other. Occasionally, however, a heterologous strain would be agglutinated to the titer limit.

A horse was immunized with a Pfeiffer bacillus strain (07) obtained from a pneumonic lung at necropsy in the 1918 influenza epidemic. Rabbits have reacted quite sharply to this strain and immunization of these animals was effected with some difficulty. After some trials an agglutinative rabbit serum of the average titer (1:800-1:1,600) was obtained, and comparison of the agglutinative action of rabbit and horse serums carried out. Normal horse serum seems to possess somewhat greater agglutinative powers for the Pfeiffer bacillus than does normal rabbit serum. Parallel tests on 48 strains of various origins with immune horse serum and immune rabbit serum showed no noticeable difference. The titer limit of the horse and rabbit serums in one experiment was 800. Only the homologous strain was agglutinated to the titer limit. The other results were as follows: Of 47 heterologous strains, horse serum (800) agglutinated 8 at 400 and 15 at 200 and rabbit serum (800) 7 at 400 and 14 at 200.

Spontaneous agglutination of Pfeiffer bacillus cultures has troubled some observers.[7] This difficulty when it occurs can be largely overcome by diminishing the amount of salt in the suspending solution to 0.2%. The best means of avoiding spontaneous agglutination, however, is to keep the cultures in a condition of good vitality by frequent transfers. Stock cultures should be transferred at least twice a week and a 24-hour culture always used for testing. The following experiment shows the effect of unsuitable methods of growth on the production of spontaneous agglutination. Sixty-five strains were kept on chocolate agar at 37 C. for 7 days and then for a few days longer at room temperature. About 50 of these strains were successfully nursed back into culture, at first usually scant. These strains had in previous tests averaged about 10% spontaneous agglutination in 0.8% NaCl solution at 55-56 for 2 days. After their subjection to the unfavorable conditions mentioned over one-half and in one experiment about two-thirds of the strains agglutinated spontaneously.

While the agglutination of homologous strains with homologous serum at intervals of a few days gives uniform results, that of the heterologous strains under the same circumstances shows considerable variation. A strain which on the first test does not show agglutination at 1:50 may be agglutinated a few days later under apparently the same conditions in a 1:200 dilution, and conversely. Some examples of this irregularity in agglutination of heterologous strains appear in table 1.

If enough tests were made practically every serum would in the course of several trials agglutinate partially or completely every heterologous strain.

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[7] Wollstein, M.: Jour. Exper. Med., 1919, 30, 555; Yabe, Brit. Jour. Exp. Med., 1921, 2, p. 127.

There is no doubt that the agglutination of various strains of the Pfeiffer bacillus with the serum of rabbits immunized against a single strain betokens a definite immunologic relationship. Our observations have been controlled by other serums. Serum of normal rabbits was frequently tested and affected the suspensions slightly, when at all. Serums of animals immunized to M. catarrhalis, B. typhosus, etc., produced no agglutination of Pfeiffer bacilli beyond an occasional trace in the lowest dilution. The "partial agglutination" of the heterologous Pfeiffer bacilli with Pfeiffer bacillus serum was in every case more coarse and distinct than the heaviest clumping produced by any of the control serums. As stated, moreover, complete agglutination of all strains of the Pfeiffer bacillus could usually be observed in dilutions 1 : 100-1 : 200 if a sufficient number of tests were made.

TABLE 1

VARIATIONS IN AGGLUTINATION OF HETEROLOGOUS STRAINS *

Strain	Serum C56NP (Rhinitis Strain) (Tests Repeated at Intervals of Few Days)			
	1st	2d	3d	4th
C56NP (rhinitis) homologous strain	800	800	—	800
GL1Tba (influenza) heterologous strain	0 (50±)	0 (200±)	120	50
GL6NPob (influenza) heterologous strain	0 (200±)	200	240	200
GL10Tbb (influenza) heterologous strain	0	0 (100±)	—	50
N9NPob (normal) heterologous strain	0 (200±)	0	—	0
N10NPoa (normal) heterologous strain	0 (200±)	200	0 (240±)	800
N22NPob (normal) heterologous strain	—	100	0	0 (200±)
R1NPoa (influenza) heterologous strain	0	200	120	100

* The readings were all made after 2 days, as stated. The designation 0 signifies no agglutination at 1:50; the ± sign after numbers in parentheses partial agglutination, and the whole numbers complete agglutination at the dilution specified, 1:800, 1:200, etc.

The immunologic relationship of the different strains is, however, not as close as that of different strains of the typhoid bacillus and some other organisms. Heterologous strains, irrespective of origin, were almost invariably agglutinated in much lower degree than the homologous. Occasionally the strains from a certain locality or more strictly from a group of more or less closely associated individuals would manifest certain immunologic resemblances. Thus the strains (13 in number) from a series of influenza cases at the Great Lakes Naval Training Station, February, 1920, although differing among themselves, as a group behaved to some extent and was agglutinated more generally by certain serums, less generally by others. For example, the serum produced by a strain from influenzal meningitis had less effect on these 13 Great Lakes strains than on those of any other group (influenza strains from Camp Grant, from Chicago, and from New York, strains from normal persons, from persons suffering with colds, etc.). On the other hand, 9 strains isolated from the nasopharynx of normal persons at Pasadena, Calif., in 1921, showed no indication of locality grouping although the strains were obtained from the throats of students who were in almost daily contact with one another. The Pasadena strains were tested with 3 serums, one prepared with a Great Lakes influenza strain, the other 2 with a Chicago rhinitis strain, control tests were made with 9 strains from miscellaneous sources. The proportion of complete, partial and negative agglutinations was substantially. the same throughout, and the Pasadena strains did not behave in any instance as an approximately homogeneous group.

The strains used in this work were derived from single colonies which were again plated out before adding the cultures to the stock. By the ordinary standards these were pure cultures. The observations we have already recorded, however, that repetitions of the agglutinative tests with the same strain and the same serum do not give identical results, suggested that perhaps the cells in a culture were not equally agglutinable. A preponderance of agglutinable or non-agglutinable races in a given culture would change the reaction correspondingly. Difficulties have been experienced by some investigators in obtaining pure cultures from material containing several strains.[1]

This possible explanation of agglutinative variations was studied in some detail. Thin suspensions of Pfeiffer bacillus culture were thoroughly shaken and then smeared over heated blood agar plates. Individual well separated colonies were transferred to culture tubes and the several subcultures tested in the usual way with the serum of immunized rabbits. A serum prepared from a Great Lakes influenza strain agglutinated with great uniformity 37 homologous substrains picked from individual colonies as described. One culture which was agglutinated less strongly than the others was suspended, thoroughly shaken and plated according to the method given in the foregoing. The 33 individual substrains obtained in this way agglutinated uniformly to the titer limit of the serum (3,200).

Cultures from some of the colonies were purified yet more carefully by 3 times successive plating. Two of these were used to immunize rabbits and the serums were tested against 45 individual colony substrains from the original stock. Agglutination occurred uniformly in all cases at dilution of 1 : 1,600-1 : 3,200. A strain isolated from a person with a common cold and one from a normal throat were similarly examined. Both showed uniformly high agglutination in all substrains.

These results so far as they go give no ground for assuming that the variations observed in the agglutination of heterologous strains of the Pfeiffer bacillus are due to the predominance of differently agglutinable races within one culture.

A possible antigenic relationship between B. pertussis and the Pfeiffer bacillus is suggested by some of our tests. Five strains of B. pertussis from different sources agglutinated in 1 : 50 or 1 : 100 dilution with serums obtained from two different rabbits immunized against a Pfeiffer bacillus strain (influenza). The serums of 2 other rabbits, one immunized against a "normal" Pfeiffer bacillus strain, the other against a "cold" strain, failed to agglutinate the strains of B. pertussis (1 : 50). Normal rabbit serum also gave negative results. Six strains of B. bronchisepticus were not agglutinated by any of these serums.

Most observers concede the greater accuracy of absorption tests over direct agglutination in attempts to determine serologic relationships.

We employed the following absorption method: The serum, mixed with an equal volume of centrifugally packed bacterial mass, was diluted to 1 : 15 with 0.8% salt solution. This mixture was heated in a 56 C. water bath for 1 to 3 hours, being frequently shaken up during this process. A 2-day incubation period was found undesirable for absorption of agglutinins by a heavy bacterial mass since so long a period impaired the serum's reactivity (table 2) even if the bacteria employed were of a totally different species. After 1 to 3 hours' incubation and after a day's storage in the refrigerator, the mixtures were centrifuged for removal of the serum dilutions.

Absorption of agglutinins by the immunizing strain was readily demonstrated by titer determinations of these serums. The homologous strain absorbed all agglutinins, homologous and heterologous. Several experiments gave similar results, those of table 2 being representative.

Absorption of the heterologous strain agglutinins, on the other hand, could not be determined by similar titer comparisons. Such agglutinative titers were generally very low to start with and were quite variable on repeated tests. It was, therefore, difficult to decide whether the titer was reduced or perhaps eliminated by absorption, whether there had been nonspecific absorption, or whether the readings happened to be lower on the test made with the absorbed serums. Simultaneous tests of various serums at a given titer offers control against these sources of error. The latter method proved superior for determinations of absorption by heterologous strains.

The 1 : 15 dilution of absorbed serum was distributed to agglutination tubes, 0.15 c c to each, and a bacterial suspension added, 0.85 c c to each. The resulting 1/100 serum dilution with bacterial suspension was heated in a 56 C. water bath and read after 24 and again after 48 hours.

Table 3, following this method, indicates that homologous and heterologous Pfeiffer bacillus agglutinins are both present to a varying degree in unabsorbed immune serum. Such agglutinins are not present, beyond an occasional trace, in serum from a nonimmunized animal. A Pfeiffer bacillus strain homologous to that producing the immune serum. absorbs both homologous and, for the most part, heterologous agglutinins. A heterologous strain absorbs agglutinins homologous to itself, and to a definite though variable degree it may absorb other heterologous agglutinins. Absorption by a heterologous strain does not remove, nor even appreciably reduce, the high titer of the serum for its homologous strain (see titer comparisons given in the table).

TABLE 2

SPECIFIC AND NONSPECIFIC ABSORPTION

Strains of Pfeiffer Bacillus Sources	Test Serum of Rabbits Immunized to Strain C 56 NP: Agglutination After					
	Absorption 3 Hours at 56 C. by			Absorption 2 Days at 56 C. by		
	C 56 NP	GL 1 Tba	B. Prodigiosus	C 56 NP	GL 1 Tba	B. Prodigiosus
C 56 NP (rhinitis)................	0	800	800	0	200	0 (p. 200)
GL 1 Tba (influenza)............	0	0	100	0	0	0
C 93 NP (normal)................	0	200	200	0	100	0 (p. 200)
O 7 (influenzal lung).............	0	0 (p. 100)	200	0	0	0

All 56 strains of Pfeiffer bacilli were tested by the absorption method, with results not differing in any essential from those recorded in the foregoing. We have sometimes observed what is ordinarily termed an immunologic relationship between strains of different origin, as illustrated in table 3 between GLITob (influenza) and C 218-2 NPoa (rhinitis). These strains were isolated in persons living in localities over 50 miles apart. No direct or indirect contact was known to exist, and is highly improbable. The influenza strain was isolated at the Great Lakes Naval Training Station in January, 1920, the rhinitis strain in Chicago in March of the same year; the latter is indol-negative, the former indol-positive. Whether a similarity of agglutinative behavior under such circumstances has much biologic, far less much epidemiologic, significance seems doubtful. So far as the Pfeiffer bacillus is concerned, the results obtained by the absorption method are no more illuminating than those obtained by direct agglutination. In a certain sense they even may be confusing, as in the apparent "immunologic identity" of the strain mentioned, and in the "immunologic independence" of 6 strains isolated from the 6 members of one family as recorded by Valentine and Cooper.[1] An interesting instance of discordant results in absorption experiments has been given by Coca and Kelley.[2]

TABLE 3

ABSORPTION OF HETEROLOGOUS STRAIN AGGLUTININS *

Source of Strains	Serum of Rabbits Immunized to Strain C 56 NP, 1:100 Dilution							Control Normal Serum	Control Suspension
	Not Absorbed	After 1½ Hours Absorption by							
		B. coli	GL 1 Tob	N 10 NPoa	C 20 NO	C 218-2 NPoa	C 56 NP		
GL 1 Tob (influenza)..	++	+++	0	++	+++	0	0	0	0
N 10 NPoa (normal)...	+	+	Tr.	0	0	Tr.	0	0	0
C218-2 NPoa (rhinitis).	+++	+++	0	++	+++	0	0	0	0
C 56 NP (rhinitis)......	+++	+++	+++	+++	+++	+++	0	0	0
GL 6 NPob (influenza)	++	++	Tr.	Tr.	Tr.	0	0	0	0
GL 8 NPoa (influenza)	+++	+++	Tr.	++	++	0	0	0	0
GL 10 Tbc (influenza)..	0	0	0	0	0	0	0	0	0
N 33 NPob (normal)...	+++	+++	0	++	..	Tr.	Tr.	0	0
C 56 NP titer..........	400	400	400	400	400	400	0	0	0

* The symbols used are to be construed as complete agglutination for +++, very pronounced for ++, pronounced for +, slight or trace for Tr. and none for 0.

We have had the same experience as other observers (Povitzky and others,[2] Utheim,[2] Maitland and Cameron[3]) in occasionally finding several agglutinatively different strains in the respiratory tract of one person. In one patient suffering from influenza we found three strains that were distinct by the agglutinin absorption test. Bell[2] has reported a similar instance. Such findings are, to be sure, exceptional. We have usually found that when several colonies of the Pfeiffer bacillus are picked from a plate made from a throat swab they are of a single "immunologic" as well as cultural type.

The phenomena observed by ourselves and others in studying the agglutinative reactions of different strains of the Pfeiffer bacillus with different immune serums may be interpreted in a relatively simple way. If we assume that the Pfeiffer bacillus represents a group of semi-parasitic or commensal micro-organisms with a predilection for inhabiting the human throat and with relatively slight invasive power we should have reason from analogy to suppose that agglutinative uniformity would be improbable. It is well known that the high agglutinative homogeneity exhibited by various strains of the typhoid bacillus is exceptional among bacterial species. The group of pneumococci comprises three or more definite or fixed types, and a large number of other strains possessed of more or less independent qualities. Pfeiffer bacilli apparently stand yet farther away from a definitely established group-pathogenicity although their invasive power under certain conditions is undoubted. The presence of bacilli of this class in the throats of a large percentage of persons in normal health has been demonstrated by the work of many observers in widely separated localities. It is, however, especially noteworthy that strains isolated from "influenzal meningitis"—which has never been proved to have any connection with influenza—manifest a distinctly greater immunologic (agglutinative) resemblance than do strains from influenzal pneumonia lesions or other sources. Rivers[8] has pointed out that a very large proportion of the meningeal strains of the Pfeiffer bacillus produce indol, thus tending toward a cultural as well as an immunologic uniformity. It is tempting to conclude, therefore, that this group of hemophilic bacilli, in general of low invasive properties, may not only possess the capacity under appropriate conditions of developing varieties with somewhat exalted pathogenic powers, but that such a variety as that exemplified in the meningeal strains is actively in process of formation. The fixing of such a pathogenic type is a process accompanied by a stabilization of cultural and immunologic characters. With this view of the significance of uniform agglutination in the life of a bacterial species as the fixing of a biologic quality in connection with the acquisition of power to invade the animal body, the biologic position of these hemophilic bacilli becomes relatively clear. The failure of ourselves and of others to find consistent immunologic reactions among the strains isolated from epidemic influenza is a strong argument against the assumption of a primary causal relation between influenza and the Pfeiffer bacillus.

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[8] Jour. Am. Med. Assn., 1920, 75, p. 1495.

SUMMARY AND CONCLUSIONS

The particular strain of Pfeiffer bacillus used to produce an immune serum is usually agglutinated by the homologous serum more rapidly, more constantly and in higher dilution than any heterologous strain.

Absorption tests throw little if any more light on the true biologic relationship of the different strains in this group than does direct agglutination.

As a rule, each strain of Pfeiffer bacillus possesses a serologic individuality.

Occasionally strains from independent sources exhibit a serologic identity. This is not common.

As many as three serologic races may be present at the same time in the throat of one patient.

There is no correlation between indol-producing powers and agglutinative affinities, except possibly in the strains isolated from meningitis.

The lack of any definite serologic grouping among the strains of Pfeiffer bacilli is an indication that a distinct invasive type, or types, has not become fixed, and is an argument against regarding any member of this group as the primary causal agent in epidemic influenza. Possibly a race of "influenza meningitis" bacilli is in process of evolution.